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Assorted effects of TGFβ and chondroitinsulfate on p38 and ERK1/2 activation levels in human articular chondrocytes stimulated with LPS

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Summary

Objectives: Inadequate cellular response of chondrocytes to stress frequently terminates in osteoarthritis (OA). Adequate response is fundamentally modulated by concerted cytokine signaling events, directing degradation and synthesis of cartilage on articular surfaces where and whenever necessary. Transforming growth factor (TGF)β is a prominent mediator in cartilage anabolism, although particular catabolic activities are occasionally reported. Clearly, before the TGFβ signal gets through to the gene regulatory machinery, cross talk with modulators occurs.

Method: We tested the hypothesis whether chondroitinsulfate (CS) modulates cell signaling. TGFβ and/or soluble CS was added to human articular chondrocytes (HACs) and activation of p38 and extracellular signal related kinase (ERK)1/2 was determined by immunoblot analysis. Expression levels of mRNA of matrix metalloproteinase (MMP)-2, -3 and -13 were determined by real-time polymerase chain reaction (PCR).

Results: No significant effects were observed unless cells were stimulated with lipopolysaccharide (LPS), invigorating catabolic metabolism in chondrocytes. LPS effects, however, were profoundly modulated by TGFβ, CS and both applied in combination. Most prominent, the silencing of p38 stress signal by CS was superimposable to that of TGFβ. Phospho-ERK1/2 levels were raised by TGFβ three-fold over LPS induced levels. In contrast, CS treatment, alone or combined with TGFβ, reduced phosphorylation significantly below LPS induced levels. Finally, suppression of LPS induced MMP-13 mRNA levels resulted with CS.

Conclusion: Soluble CS modulates signaling events in chondrocytes concurrent with MMP-13 down regulation. The effects observed suggest a feedback signaling mechanism cross talking with TGFβ-signal pathways and may serve an explanation, on the cellular level, for the beneficial effects found in clinical studies with pharmacologic application of CS.

Key words: Chondrocyte, Signal transduction, TGF, Metalloproteinases, p38, ERK1/2, Chondroitinsulfate, LPS.

Introduction

The molecular events dominantly determining induction of osteoarthritis (OA) initially seem to be linked to the locally regulated capacity to build up new cartilage within the adaptive reconstruction of the tissue. This regulation is exceptionally sophisticated permitting local repair, with degradation next to built up of cartilage on a given articular surface. Consequently, there must be a sophisticated signaling in effect which, as soon as degradation is locally initiated, serves as control for the vigor, modulates and/or terminates degradation and eventually initiates synthesis of new material. Typically, biological systems utilize a mechanism, well established as feedback control, where end products signal to the start reactions to trim the dynamics of the reaction chain.

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Within breakdown of cartilage by aggrecanase, cleavage of glycan side chains produces soluble chondroitinsulfate (CS) moieties[1,2]. This is a critical initial process in cartilage turnover, as aggrecan depletion substantially accelerates collagen digestion in cartilage[3,4]. We here tested the hypothesis that a glycan portion of aggrecan, the CSs, as occurring in initial degradation of cartilage, might fulfill such a feedback control. A clinical study described that application of soluble exogenous CS produced a protective effect in acute cartilage degradation[5], but the molecular mechanisms are obscure. In addition elevation of serum levels of CS after oral application and a pronounced tropism towards the knee articular space have been reported[6]. It is thinkable that the pharmacological elevation of CS levels in joints assists the feedback and thereby contributes to the protective effect.

Functionally outstanding among the cytokines that transduce anabolic signals into human articular chondrocytes (HACs) is transforming growth factor (TGF)β. It enhances but also silences anabolic and catabolic gene expressions. The main route of TGFβ signaling takes place via the well described SMAD pathway[7], but emerging evidence suggests intense cross talk with extracellular signal related kinase (ERK)1/2 and p38 that brings to effect the

dichotomic potency[8–10]. The dichotomic behavior suggests that TGFβ can crucially tip the metabolic balance to either side by interpretation of signal cross talk. The idea is accentuated by *in vivo* findings such as that chondrocytes upon treatment with TGFβ down regulate matrix metalloproteinase (MMP) expression levels immediately adjacent to OA lesion, whereas chondrocytes more distant from the macroscopic lesion increase MMP mRNA[11]. We therefore choose the influences of TGFβ to be measured as standard for recognized significance.

During the experiments we learned that CS cross reacts with TGFβ induced signaling and investigated this phenomenon by coincubations more closely. In the consequence of signal modulation, changes in expression levels of key MMPs would validate the meaning of signal events produced by TGFβ/CS additions. Expression and profiling studies available in literature led us to select MMP-2, -3 and -13[12] for determination of expression levels. In particular, MMP-3 is the most highly expressed MMP gene in normal cartilage and is significantly reduced in OA but nevertheless remains comparatively high, reflecting a crucial maintenance function that becomes deregulated in OA. A consistent finding in several reports of human OA, as well as in animal models, is the upregulation of MMP-2 and -9 (the gelatinases) and MMP-13 (collagenase-3)[13,14]. We included this selection of MMP key players in normal cartilage degradation, in view of the facts that deregulation is assumed to lead to OA and p38, also named stress kinase, as well as ERK1/2 signaling is documented to play a regulatory part[15–18].

To simulate a catabolic situation in cell culture, we used dedifferentiated chondrocytes in culture that were challenged by lipopolysaccharide (LPS), a bacterial cell wall product, frequently associated as a set off for OA induction[19–21]. The data show that soluble CS, added to HACs in culture, modifies signal transduction significantly in LPS-treated cultures by particular mechanisms that modulate TGFβ signaling. The data strongly support the proposed feedback mechanism and may serve to explain at a cellular level the beneficial effect of CS described in clinical studies[5,22,23].

Material and methods

ISOLATION AND MONOLAYER CULTURE OF HACs

HACs were collected with informed consent and approval of the local ethical committee as described[24] from the hips of three patients with no history of joint disease who were scheduled to undergo joint replacement after fracture of the femoral neck. Briefly, the samples of cartilage were collected in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) enriched with 50 mg/ml of gentamicin and 5 mg/ml of amphotericin B (Life Technologies). The cartilage was diced into pieces of $1-3\,mm^2$ and placed in DMEM containing 200 U/ml of collagenase (Sigma Chemical, St Louis, Missouri). The tube was placed on an orbital shaker at 25 rpm, and incubated at 37°C for 20 h. The following day, the digest was resuspended and centrifuged at 1000 rpm for 10 min. The pellet was gently resuspended with calcium- and magnesium-free phosphate-buffered saline (PBS). The suspension was filtered through a 100 mm mesh. Cells were centrifuged and washed, then counted, resuspended in medium and placed into T-75-flasks at a density of $1.5 \times 10^4/cm^2$. The growth medium for all cultures was DMEM with 450 mg/dl of glucose (Life Technologies). The medium was supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 mg/ml of streptomycin (all from PAA) and 50 mg/l of ascorbic acid (Sigma Chemical). Cells from all preparations were pooled and passaged at 80% confluence by release from the dishes with a solution of 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) (PAA) in a ratio of 1:3. All the cell cultures were grown in a humidified CO_2 incubator (Queue Systems Inc.) under 95% air and 5% CO_2 at 37°C. For experiments, cells from the eighth to the tenth passages were seeded into 6-well plates (Greiner Bio One) at a density of 1.5×10^5 cells/well and grown to confluence. Culture medium was changed 24 h prior to incubation with 1 μg/ml LPS (Sigma) and/or 10 ng/ml human recombinant TGFβ (Calbiochem) and/or 25 μg/ml CS for the indicated times. All experiments were done at least in independent triplicates unless separately indicated and at least duplicate determinations within each experiment were used to calculate the activities. Statistical significance was calculated using Graph Pad Prism's software one column *t*-test and Bonferroni's Multiple Comparison Test.

ANALYSIS OF ACTIVATION OF MAP KINASE COMPONENTS

Following activation, cells were washed three times with cold PBS and were then lysed in 70 μl lysis buffer per well[25] (10 mM Tris, pH 7.2, 150 mM NaCl, 0.5% Nonidet P 40 and 2 mM EDTA). Protease inhibitor (Sigma P8340) and phosphatase inhibitors (NaF, 5 mM and Na-ortho-vanadate 0.2 mM) were added freshly prior to use. Cell lysates were harvested with a plastic scraper, shock frozen in liquid nitrogen and thawed and then centrifuged at $13{,}000 \times g$ for 15 min at 4°C. Protein concentration was determined using a BCA protein assay (Pierce). Samples were either immediately used for Western blotting or stored at −20°C.

Sodium dodecyl sulfate (SDS) gel electrophoresis and Western blotting

The activation status of ERK1, ERK2 and p38 was assessed by Western blotting using antibodies that recognize the activated form. Cellular extracts (at least 12 μg for ERK1/2 and 15 μg for p38) were diluted in the ratio 1:4 in Laemmli buffer (62.5 mM Tris, pH 6.8, 10% v/v glycerol, 1% w/v SDS, 1% v/v β-mercaptoethanol, and 0.01% w/v bromophenol blue) and run on a 10% SDS-polyacrylamide gel in a BIO-RAD miniprotean 3 apparatus. Proteins were transferred onto Trans-Blot nitrocellulose (BIO-RAD) in blotting buffer (50 mM Tris, 192 mM glycine, pH 8.3, containing 20% v/v methanol and 0.1% SDS) for 45 min with 400 mA constant current. To verify blotting efficiency, blots were stained in Ponceau S solution (0.1% w/v Ponceau S in 5% v/v acetic acid) for 5 min and documented by photography. The straight relationship of this staining to ERK protein mass was established by probing with pan-ERK antibody signal evaluation in a series of experiments. After destaining, membranes were incubated for 1 h in blocking buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20 and 5% w/v nonfat dry milk) followed by washing buffer (25 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween). Subsequently, membranes were incubated over night with primary antibodies (anti-pERK 1/2 and p-p38 from Cell Signaling) 15 μl each in 15 ml of blocking buffer. After 3×10 min washing, the membranes were incubated for 60 min with a goat anti-rabbit peroxidase conjugated IgG antibody (BIO-RAD) diluted 1:15,000 in blocking buffer. After 3×10 min washing, antibody labeled proteins were

visualized with chemiluminescence substrate (SuperSignal, Pierce 34080) and documented using ChemiImager 4400 (Biozym) with software to compute band intensities.

GENE EXPRESSION ANALYSIS

Total cellular RNA was extracted with 1 ml of Trizol reagent per well (Invitrogen) according to the manufacturer's protocol. The RNA pellet was dissolved in 20 μl diethylpyrocarbonate (DEPC) (Sigma) treated water and reverse transcribed using random hexamer primers (Fermentas) and MuLV-reverse transcriptase (Fermentas). RNA extract (10 μl) and random hexamers (2.5 μM) were incubated for 10 min at 70°C and then the master mix was added. An incubation for 60 min at 37°C and finally for 5 min at 95°C was performed to complete the reaction.

Real-time polymerase chain reaction (PCR) was carried out using LUX® (Light upon extension) Primer (Invitrogen), Platinum® Quantitative PCR SuperMix-UDG 2× (Invitrogen) and ROX Reference Dye (Invitrogen). LUX™ Primers were designed using LUX™ Designer software available in web-based format on the Invitrogen homepage (http://www.invitrogen.com). The sequences of the primers (see below) were checked using the NCBI Blast Software and specificity of RT-PCR products was documented with gel electrophoresis and resulted in a single product with the desired length:

MMP-2 labeled (FAM) reverse	5′- cacctg TCT CTG GGT CCA GAT CAG GtG -3′
MMP-2 unlabeled forward	5′- GCA AGC CCA AGT GGG ACA A -3′
MMP-3 labeled (FAM) reverse	5′- caccag GCA TCT TTT GGC AAA TCT GGt G -3′
MMP-3 unlabeled forward	5′- AGC CCA GGT GTG GAG TTC CT-3′
MMP-13 labeled (FAM) reverse	5′- caactt CGC AGC AAC AAG AAA CAA G5TG-3′
MMP-13 unlabeled forward	5′- TGG GCC AAA TTA TGG AGG AGAT-3′
GAPDH labeled (JOE) reverse	5′- caacg CCA ATA CGA CCA AAT CCG tTG-3′
GAPDH unlabeled forward	5′- CCA CAT CGC TCA GAC ACC AT-3′

Real-time PCRs were carried out in 20 μl volumes in 96-well plates (ABI PRISM® 96-Well Optical Reaction Plates and MicroAmp® Optical Caps) using the ABI PRISM® 7700 Sequence Detection System.

The cycling conditions were chosen according to the following scheme:

UDG-treatment	2 min	50°C	
Initial denaturation	2 min	95°C	
Denaturation	15 s	95°C	\
Annealing	30 s	60°C	} 45×
Elongation	30 s	72°C	/

Quantification was performed according to the mathematical model designed by Pfaffl[26]. Briefly, for the mathematical model it is necessary to determine the crossing points (CPs) for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. Real-time PCR efficiencies were calculated, according to $E = 10^{[-1/\text{slope}]}$. The relative expression ratio (R) of a target gene is calculated based on E and the CP deviation of an unknown sample vs a control, and expressed in comparison to a reference gene.

Results

SIGNAL ACTIVATION BY TGFβ AND/OR CS IN CELLS WITHOUT LPS STIMULATION

We established the baseline values for the parameters p-p38, and pERK1/2 under normal conditions and evaluated thereafter the changes induced upon addition of TGFβ as the benchmark stimulus and compared CS effects to that. To capture direct signaling effects, we measured the respective levels at 30 min and, given the possibility that signaling occurs subsequent, with other effects invoked ahead before the signal cascades were reached, we measured activities in 72 h incubations in addition. The latter pictures the duration of the activation. The amount of activated, phosphorylated, forms of the signaling molecules was calculated from band intensities after Western blot analysis. Antibodies specific for the phosphorylated forms of p38 stress kinase (p-p38) and phospho-ERK1/2 (pERK1/2) detected protein bands at 43 kDa (p-p38) and 42 and 44 kDa (pERK1/2) which were visualized by chemiluminescence. The integrated optical densities (IODs) resulting from signal intensity analysis of the bands are presented as relative activity (IOD treated sample/IOD control). The results are grouped − TGFβ, CS, and TGFβ and CS showing effects of short term (grey bars) and long term (open bars) incubations in HACs under standard conditions (Fig. 1). With TGFβ addition, a tendency toward (not significant; $P > 0.05$) elevated pERK2 activation was observed (left panel Fig. 1). With CS incubations no effects were found (middle panel). With simultaneous coincubation of both substances, a significant elevation ($P < 0.001$) of pERK2 resulted with prolonged incubation (open bar, right panel Fig. 1).

SIGNAL ACTIVATION BY LPS STIMULATION

A profound benefit of CS was found preferentially in conditions of florid OA and we modeled this catabolic situation by incubation with LPS. Chondrocytes were incubated with LPS and subjected to Western blot analysis as described above. The IODs resulting from signal intensity analysis are presented as relative activity (IOD LPS-treated sample/IOD control). In Fig. 2 it can be seen that the treatment with LPS invigorated p-p38 stress kinase immediately (grey bars) and the effect persisted for at least 72 h at the same level (open bars). Also both pERK1 and 2 were elevated, although for short duration only (grey bars). In detail, p-p38 was elevated 3.5-fold at 30 min and 72 h ($P < 0.001$) and pERK1 and 2 were elevated two-fold ($P < 0.05$) at 30 min only.

ACTIVATION OF MMP EXPRESSION LEVELS BY LPS

In Fig. 3 the expression levels of MMP-2, -3 and -13 were assessed by real-time PCR and the ratios of the values resulting from LPS treatment vs control treatment values are shown. MMP-3 and -13 mRNA levels were significantly induced, while MMP-2, considered to be constitutively expressed, remained unchanged. In detail, we found that incubation with LPS increased the expression levels of MMP-3 more than six-fold ($P < 0.01$) and MMP-13 more than nine-fold ($P < 0.01$) after a 24 h treatment. These

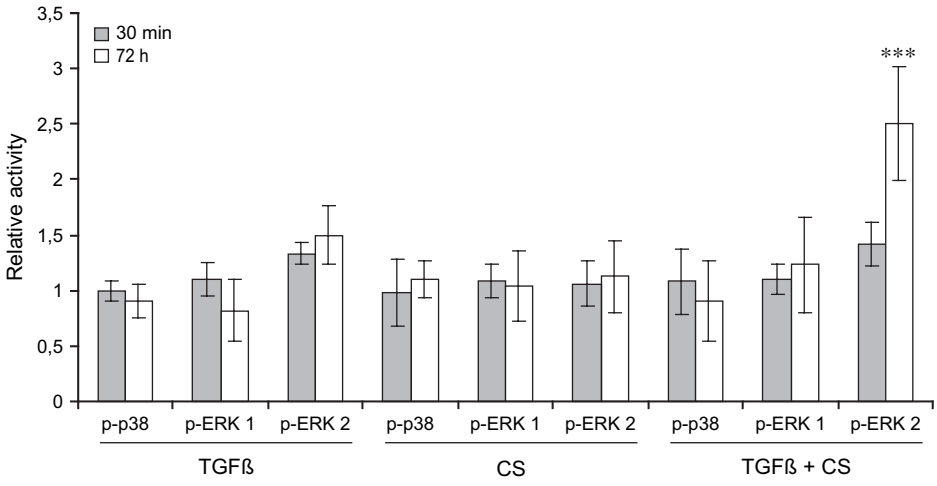

Fig. 1. Short and long term effects of TGFβ and CS on the activity levels of p38 and ERK1/2. HACs were incubated for the indicated time periods (30 min and 72 h) with human recombinant TGFβ (10 ng/ml) or CS (25 μg/ml) or both, in DMEM containing 10% FCS. Activation levels of p38 and ERK1/2 were determined by immunoblot using phosphospecific antibodies. Equal sample loading was assessed by BCA™ Protein Assay Kit and controlled by Ponceau S staining. Band intensities were calculated using the ChemiImager 4400 (Biozym). Data are presented as relative change to control activity (IOD-treatment/IOD-control). Each bar represents the mean of three independent experiments (performed in duplicates) ± SD (vertical lines). Significant changes to control: *** = $P < 0.001$ (Bonferroni's Multiple Comparison Test).

results accentuate the shift towards a catabolic state in the HAC cultures, induced by incubation with LPS.

SIGNAL MODULATION BY TGFβ AND/OR CS IN CELLS STIMULATED WITH LPS

With the determinations of signal levels after LPS stimulation in hand, we now determined the modulation of these by addition of TGFβ and CS or both in combination. Changes induced by the indicated treatments are given as percentage of induction (positive numbers) and reduction (negative numbers). The IOD resulting from the LPS challenge now serves as the basic value. Without the addition of LPS (Fig. 1) we found that TGFβ as well as CS did not change the activity of p38. But, as can be seen in Table I, TGFβ and/or CS reduced stress kinase p-p38 in LPS conditioned HACs. A trend towards reduction was observed immediately (30 min) and reached significance after 72 h. In detail, TGFβ reduced p-p38 to control level (−100%, $P < 0.001$), that is the levels found in cells grown without LPS, and CS additions resulted in a 54% reduction ($P < 0.01$). The combined addition of both produced the same effect (−59%, $P < 0.01$).

With regard to ERK, the effects of the treatments were fundamentally different. TGFβ produced an immediate statistically significant boost of ERK1 phosphorylation (260%, $P < 0.001$) that subsided after 72 h. ERK2 phosphorylation

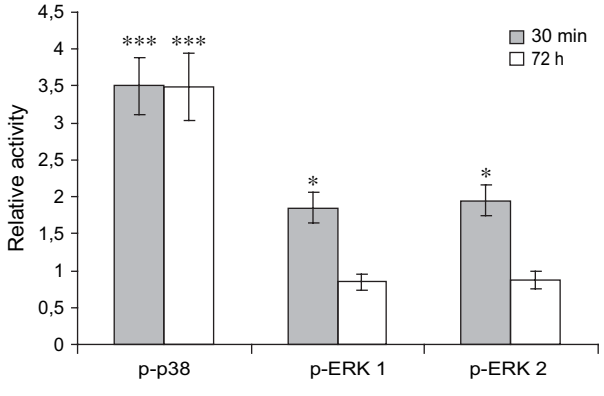

Fig. 2. Activity of p38 and ERK1/2 in HACs treated with LPS. HACs were incubated with LPS (1 μg/ml) for 30 min and 72 h in DMEM containing 10% FCS. Activation levels of p38 and ERK1/2 were determined by immunoblot using phosphospecific antibodies. Equal sample loading was assessed by BCA™ Protein Assay Kit and controlled by Ponceau S staining. Band intensities were calculated using the ChemiImager 4400. Data are presented as relative change to control activity (IOD-treatment/IOD-control). Each bar represents the mean of three independent experiments (performed in duplicates) ± SD (vertical lines). Significant changes to control: * = $P < 0.05$, *** = $P < 0.001$ (Bonferroni's Multiple Comparison Test).

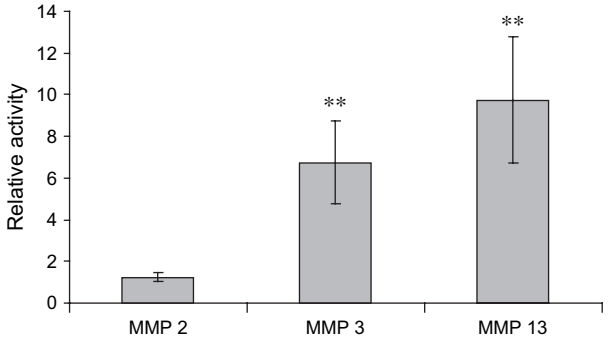

Fig. 3. Expression levels of MMPs 2, 3 and 13 in HACs treated with LPS. HACs were incubated with LPS (1 μg/ml), for 24 h in DMEM containing 10% FCS. The expression levels of MMPs and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by RT-qPCR using the LUX Primer system. The MMP expression levels were calculated according to the mathematical model of Pfaffl (normalized to GAPDH) and presented as ratios LPS-treated vs control. Each bar represents the mean of six independent experiments (performed in triplicates) ± SD (vertical lines). Significant changes to control: ** = $P < 0.01$ (one-sample *t*-test).

Table I
Changes of activity levels of MAP kinases (p38 and ERK1/2) in LPS stimulated HACs treated with TGFβ and/or CS

	Treatment					
	30 min			72 h		
	p-p38	p-ERK 1	p-ERK 2	p-p38	p-ERK 1	p-ERK 2
TGFβ	−25 (±14)	260 (±38)***	317 (±40)***	−100 (±18)***	0 (±25)	160 (±25)***
CS	−35 (±15)	−55 (±18)**	−73 (±14)***	−54 (±12)**	10 (±10)	62 (±15)***
TGFβ + CS	−38 (±17)	−100 (±25)***	−100 (±20)***	−59 (±25)**	120 (±15)***	124 (±25)***

LPS (1 μg/ml) stimulated HACs were incubated with human recombinant TGFβ (10 ng/ml) and CS (25 μg/ml) or both combined, for the indicated time points (30 min and 72 h) in DMEM containing 10% fetal calf serum (FCS). Activation levels of p38 and ERK1/2 were determined by immunoblot using phosphospecific antibodies. Equal sample loading was assessed by BCA™ Protein Assay Kit and controlled by Ponceau S staining. Data are presented as % change in activity compared to LPS treatment. Means were calculated from three independent experiments (performed in duplicates ± SD). Significant changes to LPS treatment: ** = $P < 0.01$; *** = $P < 0.001$ (Bonferroni's Multiple Comparison Test).

was also stimulated immediately (317%, $P < 0.001$), but persisted (160%, $P < 0.001$). In contrast, an immediate reduction of pERK1 (−55%, $P < 0.01$) and pERK2 (−73%, $P < 0.001$) resulted from the addition of CS. After 72 h the reduction of pERK1 vanished and pERK2 was significantly elevated (62%, $P < 0.001$). Remarkably, the combined application of TGFβ and CS produced the CS type effect: an immediate significant reduction of pERK1 and 2 (−100%, $P < 0.001$). The reduction was converted into a significant stimulation after 72 h (both more than 120%, $P < 0.001$). Together, the results of the experiments summarized in Table I uncover a cross reference of signals produced by TGFβ and CS.

RESPONSE OF LPS INDUCED MMP EXPRESSION LEVELS TO CS TREATMENT

The disclosure of mutual and divergent signaling effects supports the assumption that CS cross talks with TGFβ effects and likely is able to modulate essential metabolic processes, e.g., MMP expression, that are necessary for the maintenance of cartilage integrity. We therefore tested whether MMP expression, that was induced by LPS treatment (see Fig. 3) can be influenced by incubation with CS. In Fig. 4 the expression levels of MMP-2, -3 and -13 were assessed by real-time PCR and the ratios of the values resulting after 24 h treatment with LPS and CS are given as relative activity to the ones found in LPS-treated cells. In a series of real-time PCR experiments we found that incubation with CS significantly decreased the expression levels of MMP-13 (−30%, $P < 0.05$).

Discussion

Given that clinical studies show a benefit when CS is taken orally, we were interested to elucidate the molecular details that underlie this accomplishment. CS is believed to provide building blocks for synthesis of proteoglycans. The evidence that the effect is more significant in acute stages suggests an additional process. We here examined the hypothesis that early breakdown products of cartilage glycosaminoglycans, set free in cartilage remodeling, create feedback signals to the chondrocytes enabling a fine tuning of the process. We reasoned that the soluble CS generated in the initial phase of aggrecan breakdown, is a key candidate that can migrate to and interact with molecular sensors on neighboring cells.

When incubating cells with CS we initially found no noticeable effects on signal transduction. This might be due to unresponsiveness of our cell model composed of dedifferentiated chondrocytes or might be a physiological feature of quiescent chondrocytes. We are currently investigating the respective responses in primary differentiated HACs. Next, we challenged the cells with LPS, a bacterial cell wall product, described to induce a catabolic state in chondrocytes[19–21]. This treatment resulted in significant elevation of stress signals and subsequent rise in MMP expression. In this stage, the cells were highly responsive to CS treatment. Stress kinases as well as MMP-13 were reduced significantly and thus the overall result can be quoted as silencing of the catabolic state.

Our results also demonstrate that the impact of CS on HAC signaling is in the magnitude of TGFβ efficacy. TGFβ typically modified the LPS induced catabolic changes, but with CS and TGFβ combined, the responses were altered significantly. We found synergism and antagonism of CS on TGFβ effects. It will be of importance to elucidate the direct molecular targets for CS on the cells, be it membrane receptors or its involvement in cytokine trapping, that produce the signaling effects. Besides acting as structural components of the extracellular matrix and anchoring cells to the matrix, both extracellular and cell-surface

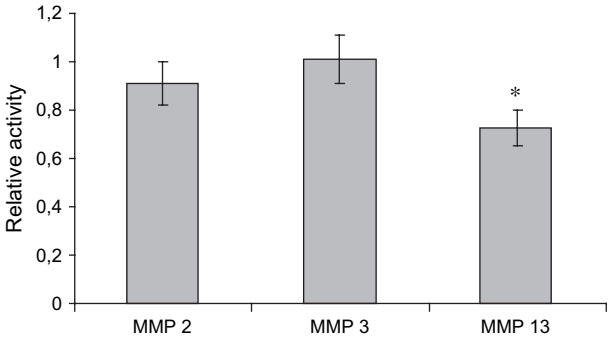

Fig. 4. Expression levels of MMPs 2, 3 and 13 in HACs treated with LPS and CS. HACs were incubated with LPS (1 μg/ml) and CS (25 μg/ml) for 24 h in DMEM containing 10% FCS. Expression levels of MMPs and GAPDH were determined by RT-qPCR using the LUX Primer system (Invitrogen). The MMP expression levels were calculated according to the mathematical model of Pfaffl (normalized to GAPDH) and presented as relative change to LPS treatment (ratios of levels of LPS and CS treated/LPS treatment). Each bar represents the mean of six independent experiments (performed in triplicates) ± SD (vertical lines). Significant changes to LPS treatment: * = $P < 0.05$ (one-sample *t*-test).

proteoglycans also bind many protein growth factors, among them TGFβ. Often, matrix-bound growth factors are resistant to degradation by extracellular proteases and so serve as a reservoir. During tissue growth and remodeling or after infection active growth factor is released by hydrolysis of the glycosaminoglycan chains. Moreover, the core protein of a cell-surface proteoglycan called betaglycan binds TGFβ and then presents it to TGFβ receptors. These examples illustrate the function of glycosaminoglycans as extracellular hormone reservoirs and mediators of binding of cytokines to their cell-surface receptors. Soluble CS might interfere with such functions and thus triggers altered intracellular signaling. So far our data fit to support the hypothesis that the soluble CS, that is locally generated in cartilage remodeling, assists in metabolic decision making in chondrocytes and tunes the effects of TGFβ. The data are also in agreement with clinical studies that indicate a more pronounced beneficial effect of oral CS application in severe cases of OA, presumably by antiinflammatory action[27].

To this end the reported experiments all support the hypothesis of a regulatory feedback potency of CS that may substantially assist the effect of substituting CS to provide nutritional supply for synthesis of CS proteoglycans, the main constituent of cartilage. Yet, we are far from understanding critical details of the mechanism.

More direct support for our assumption is currently investigated. In view of reports indicating that aggrecanase-1 is induced by cytokines, especially TGFβ[28], we are interested to examine whether a similar feedback occurs in this system analogous to the MMP.

Numerous reports demonstrate metabolic effects of CS on cells, that are often decidedly specific for the sulfation position. We recently reported that soluble CSs induce a pronounced growth modulation on length and fasciculation of neurites in primary cultured neurons[29]. Since there is a remarkable match in the use of certain signaling mechanisms in brain cells and chondrocytes we were incited to scrutinize the possibility that a sulfation type dependent interaction can as well occur in chondrocytes by CSs[10,30].

We are thus currently analyzing preliminary evidence that different sulfation patterns in CS types produce variable signaling interference, with the high C6S forms being more effective in reduction of MMP-13. In view of the major risk factor for OA, age, the concomitant significant decrease of the C6S/C4S ratio in synovial fluid is a remarkable discovery[31,32]. An examination of the levels of CS in synovial fluid of a collection of patients with staged OA (Kellgren and Lawrence scale I to IV) revealed a decline of C6S/C4S ratio from 5.6 at stage I to 2.7 at stage IV[33]. Such changes would, accordingly, predict that the feedback strength will decline with age and may thus present a new factor contributing to the elevated risk of OA with age, which could be prevented by substituting C6S.

Anyhow, the direct molecular targets for CS that produce the signaling effects need to be elucidated. This knowledge will not only help to adequately streamline the application of CS for pinpointed efficacy but will uncover important mechanisms of the regulation of cartilage growth and maintenance.

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